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Naked DNA and Adenoviral Immunizations for Immunotherapy of Prostate Cancer: A Phase I/II Clinical Trial

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Key Words

Naked DNA · Plasmid · Recombinant adenovirus · Expression vector · Prostate specific antigen · Prostate-specific membrane antigen · DNA immunization · Immunotherapy · CD86 · Granulocyte macrophage-colony stimulating factor

Abstract

Introduction and Objectives: Animal studies have indicated that the use of syngeneic dendritic cells that have been transfected ex vivo with DNA for tumor-specific antigen results in tumor regression and decreased number of metastases. Additional studies have also suggested the possibility to modulate the dendritic cells in vivo either by 'naked' DNA immunization or by injecting replication-deficient viral vectors that carry the tumor-specific DNA. Using the prostate-specific membrane antigen (PSMA) as a target molecule, we have initiated a clinical trial for immunotherapy of prostate cancer. The primary objective of the study was to determine the safety of the PSMA vaccine after repeated intradermal injections.

Methods: We have included the extracellular human PSMA DNA as well as the human CD86 DNA into separate expression vectors (PSMA and CD86 plasmids), and into a combined PSMA/CD86 plasmid. In addition, the expression cassette from the PSMA plasmid was inserted into a replication deficient adenoviral expression vector. Twenty-six patients with prostate cancer were entered into a phase I/II toxicity-dose escalation study, which was initiated in spring 1998. Immunizations were performed intradermally at weekly intervals. Doses of DNA between 100 and 800 μg and of recombinant virus at 5×10^8 PFUs per application were used.

Results and Conclusion: No immediate or long-term side effects following immunizations have been recorded. All patients who received initial inoculation with the viral vector followed by PSMA plasmid boosts showed signs of immunization as evidenced by the development of a delayed-type hypersensitivity reaction after the PSMA plasmid injection. In contrast, of the patients who received a PSMA plasmid and CD86 plasmid, only 50% showed signs of successful immunization. Of the patients who received PSMA plasmid and soluble GM-CSF, 67% were immunized.

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However, all patients who received the PSMA/CD86 plasmid and sGM-CSF became immunized. The patients who did not immunize during the first round were later successfully immunized after a boost with the viral vector. The heterogen ity of the medical status and the presence in many patients of concomitant hormone therapy does not permit unequivocal interpretation of the data with respect to the effectiveness of the therapy. However, several responders, as evidenced by a change in the local disease, distant metastases, and PSA levels, can be identified. A phase II clinical study to evaluate the effectiveness of the therapy is currently underway.

Introduction

Two recent discoveries in basic immunology have made the prospect of immunotherapy of cancer less distant. The first discovery relates to the nature of the antigens that are recognized by T cells, the likely effectors in cancer immunotherapy [1–5]. T cell receptors react with antigen-derived peptides previously bound to major histocompatibility (MHC) molecules. Specialized antigen-presenting cells (APCs) present peptides from extracellular antigens in association with class II molecules, while parenchymal (and cancer) cells express class I MHC molecules displaying peptides from intracellularly synthesized proteins [6, 7]. Different peptides may bind to one and the same MHC molecule thus creating different 'bulging away' antigenic specificities [4, 5].

All parenchymal cells, as well as most tumor cells, express membrane-bound, peptide-occupied MHC class I molecules as a constant display of their most recent biochemical activity. A 'cancer', T-cell-recognized antigen, therefore, involves expression of peptides from tumor-associated antigen(s) bound to a class I MHC molecule and presented on the tumor cell membrane [8–10]. Several groups of tumor-associated antigens have been identified such as products of completely silent genes (MAGE, RAGE) [11, 12], differentiation antigens (gp 75, CEA) [13, 14], antigens resulting from mutations (bcr-abl in myeloid leukemias) [15], high-density normal antigens (PRAME, Her-2/neu, p53) [16–18] or viral antigens (E7 oncoprotein from human papilloma virus) [19].

Tissue-specific antigens can also serve as targets for cancer immunotherapy. Prostate cells provide a particularly useful model for the testing of this strategy since the application to prostate cancer does not mandate discrimination between normal and malignant cells. Possible products that can be targeted are the prostate-specific membrane antigen (PSMA) [20], the prostate-specific antigen (PSA) [21, 22] and prostate acidic phosphatase [23].

The second discovery focuses on the initiation of the immune responses and postulates that effective stimulation of resting naive and memory T cells by an APC requires at least two separate signals [24–27]. Both of these signals originate from the APC. The first is delivered from the recognition of the antigen itself and, if not accompanied by the second, results in T cell paralysis or death [25]. The provision of the second signal, therefore, is essential for T cell stimulation and expansion [28]. This costimulatory signal results from binding of a member of the B7 family of ligands (CD80, CD86), expressed on the APC, to CD28 on the surface of T cells [29–31].

In order to initiate an immune response, therefore, a cancer antigen must be presented to naive T cells by professional APCs [32]. The most efficient APCs in vivo are the dendritic cells (DC) [33]. DC are present in tissues and, when alerted by 'danger signals', they mature, accumulate antigen and migrate to the regional lymph nodes [28, 34]. A colony-stimulating growth factor, called granulocyte-macrophage colony stimulating factor (GM-CSF) has been found to act as a danger signal [35, 36].

The myeloid DCs originate from a bone marrow CD34+ precursor common to granulocytes and macrophages [33]. A CD14+ intermediate has been described in peripheral blood with the potential to differentiate along the DC or the macrophage pathway under distinct cytokine conditions [37, 38]. Depending on a concentration gradient in the milieu, peptides derived from tumor-associated antigens can be loaded onto the MHC molecules of DC simply by incubation in vitro [39, 40] and recently, trials using this approach have been initiated [20, 41].

Alternatively, DC can be genetically engineered to produce tumor-associated antigens and, respectively, peptides derived from them [42]. Such genetic engineering can be accomplished either in vitro (transfection with plasmids or viral vectors) [42] or in vivo ('naked' DNA immunization, immunization with viral vectors) [43–45]. DNA vaccination has proven to be an effective means of immunization against various noxi, including malaria [46], influenza [47], rabies [48], herpes simplex [49], leishmaniasis [50], tuberculosis [51] and borrelia infection [52], and may have a potential for prevention or treatment of autoimmune diseases [53] or allergies [54]. The immunogenicity of plasmid DNA

is enhanced by short-term sequences that contain CpG dinucleotides in particular base contexts [55–57]. Several reports have shown that injection of naked DNA induces both humoral and cellular immune responses directed against the encoded immunogenic proteins [58]. The development of cytotoxic (T1) T lymphocyte responses is particularly important against virally infected cells or tumor cells. Experimental work in animals has shown that the intensity of the immune response can be increased by the introduction of sequences encoding for costimulatory molecules (CD86) [59] or soluble lymphokines (GM-CSF and IL-12) [36, 60], although the mechanisms involved in T cell activation by DNA immunization are not completely characterized.

Several groups have recently reported on the safety and biologic effectiveness of DNA vaccines for immunizations against HIV and malaria [22, 61]. Use of PSA-recombinant vaccinia virus was also reported to be safe and to lead to an immunologic response [22]. The following phase I/II safety/dose-escalation clinical study was conducted on prostate cancer patients. We have chosen to work with PSMA as our target antigen and for that purpose the DNA encoding the extracellular portion of the PSMA was inserted in both a plasmid expression vector and in a recombinant, replication-deficient adenovirus. The costimulatory requirements for DNA immunization in terms of the CD86 coexpression and the addition of soluble GM-CSF to the vaccine were also tested. The safety of CD86 coexpression was tested initially by injection of a separate plasmid containing DNA for human CD86 and later, by injection of a complex plasmid containing DNAs for the extracellular portion of human PSMA and for CD86.

Materials and Methods

cDNAs and Plasmids

The cDNA encoding the extracellular portion of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, Calif., USA) after amplification by RT-PCR of total mRNA, that was obtained from the human prostate cancer cell line LNCaP (CRL 1740, ATCC). The complete human CD86 cDNA was cloned into the pCR2.1 vector by RT-PCR of total mRNA, isolated from human monocytes of a healthy donor.

Both cDNAs were subcloned into a modified mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, Calif., USA) after deletion of the neomycin resistance gene. Both the XC-PSMA and CD86 plasmids contain the corresponding cDNAs under the regulation of a CMV promoter and a bovine growth hormone polyadenylation signal. The combined PSMA/CD86 plasmid contains both the XC-PSMA and CD86 DNAs, each under a separate CMV promoter and a polyadenylation signal.

Both the PSMA and the CD86 plasmids can be expressed in mammalian cells following transfection (data not shown).

The plasmid-DNA product specifications included endotoxin content below 0.1 EU/μg of DNA; lack of detectable amounts of bacterial RNA, genomic DNA or ssDNAs as determined by agarose-gel electrophoresis; and less than 10 μg of protein/mg plasmid DNA as determined by colorimetric assay (Bio-Rad, Hercules, Calif., USA).

Prior to injection, the plasmids were diluted with sterile pyrogen-free saline.

Adenovirus

The entire expression cassette from the XC-PSMA plasmid described above was inserted in a replication-deficient (E1, E3 deletions) adenoviral (Ad5) vector (Quantum Biotechnologies, Toronto, Canada). The resulting adenovirus Ad5-PSMA is a replication-deficient recombinant adenovirus in which the replication-essential genes E1 and E3 are replaced with the expression cassette containing a coding sequence for XC-PSMA.

Prior to injection, the Ad5-PSMA was diluted with sterile pyrogen-free saline.

Growth Factors

Soluble GM-CSF (sGM-CSF, Leukine, Sargramostim) is a recombinant human GM-CSF produced by recombinant DNA technology in a yeast expression system (Immunex, Seattle, Wash., USA).

For injection GM-CSF was reconstituted and diluted according to the manufacturer's guidelines.

Study Design

The study was conducted in accordance with the Bulgarian National Drug Institute IND and approved on October 22, 1997. All patients signed an informed consent form before admission into the study. Data from monitoring visits were shared with the patients as the study proceeded, and the patients were reminded that they were free to withdraw from participation at any time.

A total of 26 patients were included in the study. All patients were immunized intradermally around the naval area. An initial group of 16 patients received two inoculations of 100 μg plasmid(s) at 1-week intervals, with or without 40,000 IU sGM-CSF. All patients who received sGM-CSF were injected with 40,000 IU sGM-CSF at the same site on day 2 following each plasmid application.

The first 4 patients included into the study received both the PSMA plasmid and the CD86 plasmid but no sGM-CSF. A second group of 6 patients was injected with a cocktail of the PSMA plasmid and sGM-CSF. A third group of 3 patients received the PSMA plasmid, the CD86 plasmid and sGM-CSF at the same inoculation site. Finally, a fourth group of 3 patients received a cocktail of the combined PSMA/CD86 plasmid with sGM-CSF.

One week following the second immunization, all patients were challenged intradermally with 100 μg of PSMA plasmid around the naval area. The delayed-type hypersensitivity (DTH) response at the injection site was measured 6, 12, 24, and 48 h later.

Ten weeks after the initial inoculation, all 16 patients, and a new group of 10 patients, received an intradermal injection with 5×10^8 PFU of the recombinant Ad5-PSMA. The 16 prior patients, and 7 of the new patients, were additionally immunized twice at weekly intervals with 100 μg of PSMA/CD86, and 40,000 IU GM-CSF. The other 3 patients from the new group received two more immunizations with 5×10^8 PFU of Ad5-PSMA, each 1-week apart.

After an additional 2.5 months, all patients were again tested for DTH against the PSMA plasmid. Subsequently, all patients have been on regular boosts, at 3-week intervals, with either the PSMA/CD86

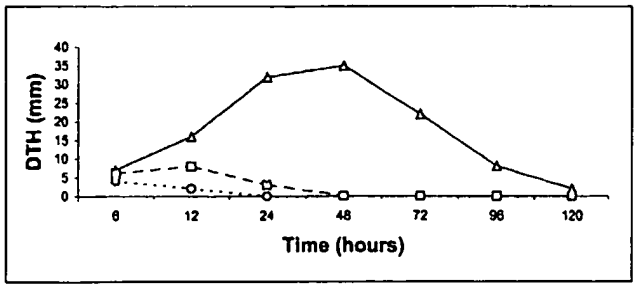

Fig. 1. DTH reaction in a prostate cancer patient at different time points following intradermal injection of either empty plasmid vector (O), sGM-CSF (□) or PSMA plasmid +sGM-CSF (Δ).

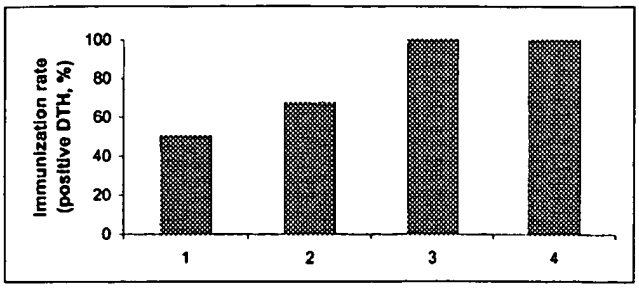

Fig. 2. DNA immunization – effect of CD86 and GM-CSF on immunization rate (development of DTH response 24 h following third PSMA plasmid application). 1 = immunization with PSMA plasmid and CD86 plasmid (n = 4); 2 = Immunization with PSMA plasmid and sGM-CSF (n = 6); 3 = immunization with PSMA plasmid, CD86 plasmid and sGM-CSF (n = 3); 4 = immunization with PSMA/CD86 combined plasmid and sGM-CSF (n = 3).

plasmid and sGM-CSF or with the Ad5-PSMA virus, some of them for > 1 year.

The 24-hour DTH reaction was recorded following each reimmunization, the aim being to maintain an intense response following each application. Depending on the intensity of the DTH response, patients' doses for the following boost have varied from 100 to 800 μg of DNA. The viral dose, whenever virus was applied, has always been 5×10^8 PFUs.

All vaccines were administered at the Urology Ward of the St Ann University Hospital, Sofia, Bulgaria. Constant monitoring of the clinical state and the vital signs was carried out for 2 h after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 h (and 48 h in the case of GM-CSF inoculation) later.

Monitoring Studies

Standard laboratory tests included CBC, urinalysis, liver enzymes, antinuclear antibodies, erythrocyte sedimentation rate and PSA. Each patient had a pelvic computer-assisted tomography scan, chest radiograph and a cardiograph on entry and on week 20 (week 10 for the 10 patients immunized with virus only). Additionally, analysis of HLA DR+; CD4+, CD8+; CD3–/CD16+CD56+; CD3+; CD11b+; CD25+, and CD19+ cells as well as the CD4/CD8 ratio, prior to, and following immunotherapy, were performed by flow cytometry. Safety was defined as lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions, as well as evidence of antinuclear or anti-double-stranded DNA antibody.

Results

Safety

All immunizations were well tolerated. No changes in vital signs occurred following injections or on follow-up visits. No significant changes occurred in erythrocyte sedimentation rate, complete blood count, serum creatinine or other blood chemistries, or urinalysis. Serum liver chemistry values remained within the normal range in all subjects. No significant changes in the analysis of CD/HLA DR+; CD4+; CD8+; CD3–/CD16+CD56+; CD3+; CD11b+; CD25+, and CD19+ cells as well as the CD4/CD8 ratio, prior to, and following immunotherapy were detected. No subject developed abnormal vital signs following injection, no significant increases in antinuclear antibody titer were observed, and no anti-double-stranded DNA antibodies were detected. One patient had a papular urticaria-like rash, with small petechiae at the center which developed 24 h after the last plasmid immunization. This rash disappeared after discontinuation of the antibiotic therapy that he was receiving for an unrelated condition. One patient had a vesicular rash after the third viral immunization. The rash was located on the back, and it resolved in the next 2 days without treatment.

Immunization Rate

A normal healthy volunteer, together with 2 patients received intradermally, at two separate sites, 100 μg of empty pcDNA3.1 vector and 40,000 IU of soluble GM-CSF. There was no DTH-like reaction at either of the application sites 24 and 48 h after the injection. Similarly, the first application of PSMA or PSMA/CD86 plasmids, with or without GM-CSF, in the other patients resulted in no reaction.

In contrast, in some patients, the second, and especially the third PSMA or PSMA/CD86 plasmid application, led to a measurable in vivo DTH response with an infiltrate that peaked with in 48 h and in some instances exceeded 40 mm (fig. 1). Using this DTH-like response we could identify patients who were immunized against PSMA.

All patients who received sGM-CSF together with both the PSMA and the CD86 plasmids, or the complex PSMA/CD86 plasmid, developed positive DTH following the third plasmid application (fig. 2, groups 3 and 4). In contrast, only 2 of the 4 patients who received the plasmids without sGM-CSF, and 4 of the 6 patients immunized with-

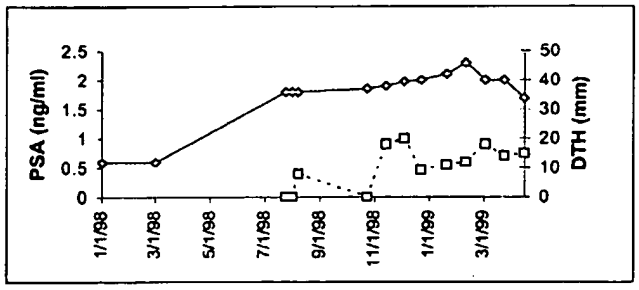

Fig. 3. PSA (◇) and DTH (□) values for patient 17 (age 67) after prostatectomy. The patient had a 2-month doubling time of PSA before the onset of immunization. Immunizations were initiated in July 1998. His PSA values have been stable for almost 1 year. The patient is on immunotherapy only.

out the CD86 plasmid, developed positive DTH following the third plasmid application.

In addition, all 10 patients who received an initial immunization with the Ad5-PSMA became immunized and developed positive DTH following PSMA plasmid inoculation. Of the 4 patients who did not immunize in the first series (fig. 2, groups 1 and 2), all became immunized following immunizations with Ad5-PSMA.

Effect of Therapy

This was a phase I/II safety-dose escalation study. The relatively small patient population varied widely with respect to their disease status and prior or concurrent treatment so that extreme caution must be exercised in judging the effectiveness of the vaccine therapy, particularly in patients receiving concurrent hormone therapy. Disease improvement, when present, was implied by local tumor regression, a fall in PSA in patients not receiving hormone therapy, and a decrease in bone pains when bone metastases were present. The 26 patients can be divided into two main groups:

Patients Who Had Prior Radical Prostatectomy

Six patients had a radical prostatectomy before entering the study. *Patients with biochemical recurrence and no detectable lymph node or bone metastases:* 3 patients presented with biochemical recurrence (rising PSA values) 1.5–3.5 years following radical prostatectomy. They were all treated solely by immunotherapy. PSA values in all of them have stabilized between 1 and 2 ng/ml following the onset of immunization (fig. 3), although it is not rare to observed such evolutions in untreated patients. Currently, they have no complaints and are considered to have stable disease. A representative case is shown in figure 3.

Patients with detectable metastases: the other 3 patients had detectable metastatic disease – 1 with distant lymph node involvement and 2 with bone metastases. They were all installed on combined hormone and immune therapy. Two of the patients were not influenced by the therapy. The 3rd patient had a PSA of 30 ng/ml and severe bone pains. He was on combined hormone and immune therapy for four months and had been on immunotherapy only for the last 7 months. His PSA has been maintained at 6 ng/ml and he has no bone pain or local disease symptomatology.

Patients with No Prior Radical Prostatectomy

Twenty patients with no prior radical prostatectomy were admitted into the study.

Patients Who Underwent Radical Prostatectomy following Combined Hormone and Immune Therapy. Four of the 20 patients had advanced local disease and received adjuvant immuno- and hormonotherapy prior to surgery. DRE performed 1 day before surgery on 3 of them, who had been on therapy longer than 3 months, revealed no palpable gland. On surgery the glands appeared significantly shrunken. The urologist also noted in these 3 cases an increased number of newly formed blood vessels lying at the fibromuscular layer. These vessels had walls that were easily injured and had increased tendency for bleeding. All coagulation tests in these patients were otherwise normal. All four patients remained on immunotherapy following prostatectomy. Three of them, however, have now (3–6 months following surgery) evidence of biochemical recurrence. A representative case from this group is shown on figure 4.

Patients with No Radical Prostatectomy or Bone Metastases. Nine patients had advanced local disease but no evidence of metastatic disease. *Patients on combined hormone and immune therapy:* 7 patients, aged 63–74 years, from this group were on combined hormone and immunotherapy. They all improved following treatment. One patient who had significant improvement locally (prostate gland shrinkage, no obstructive voiding symptoms) was removed from hormone therapy starting in February 1999, and remained on immunotherapy only. Currently, 5 months following discontinuation of hormone therapy, he has no evidence of local recurrence or metastatic disease (fig. 5).

Patients on immunotherapy only: 2 patients were on immunotherapy only. The 1st patient was not influenced by immunotherapy, had evidence of local disease progression and has recently been started on hormone therapy. The 2nd patient, however, responded to the therapy with tumor shrinkage and drop in PSA from 13 to 4.5 ng/ml and is considered a responder (fig. 6).

Patients Who Had Not Undergone Radical Prostatectomy and Who Had Metastatic Disease: The last 7 patients had metastatic disease, and no prior surgery. One had distant lymph node involvement, and 6 had bone metastases.

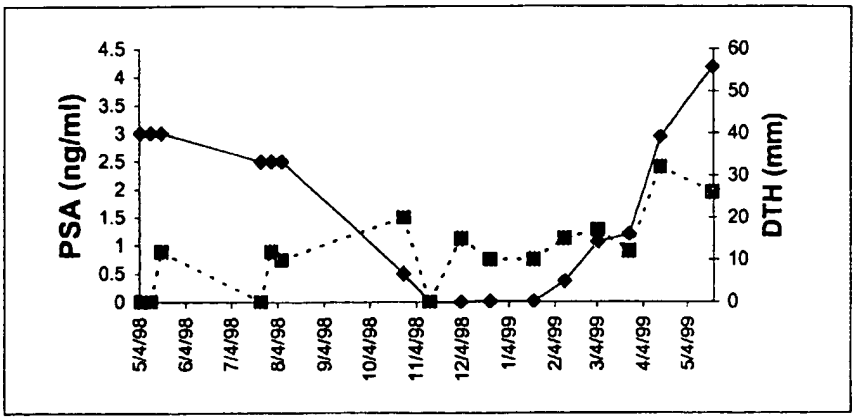

Fig. 4. PSA (◆) and DTH (■) values of patient 5 (age 58; diagnosis 12/97; $T_{3c}N_0M_0$). Hormone therapy (Zoladex, Casodex) was initiated in 1/98, immunotherapy in 5/98. No tumor was palpable at the time of prostatectomy (11/11/1998). PSA became detectable in mid-February 1999 and it has been rising steadily despite immunotherapy.

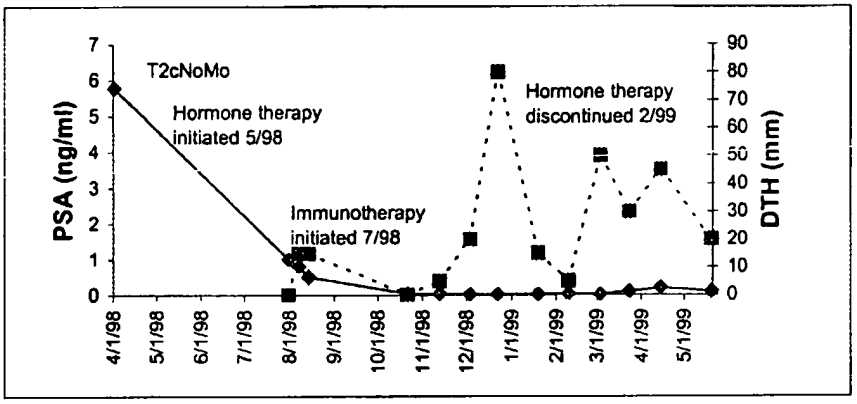

Fig. 5. PSA (◆) and DTH (■) values of patient 16 (age 63). Diagnosis in 5/98 $T_{2c}N_0M_0$. Currently no tumor but a small remnant of the prostate gland is palpable, no obstructive voiding symptoms.

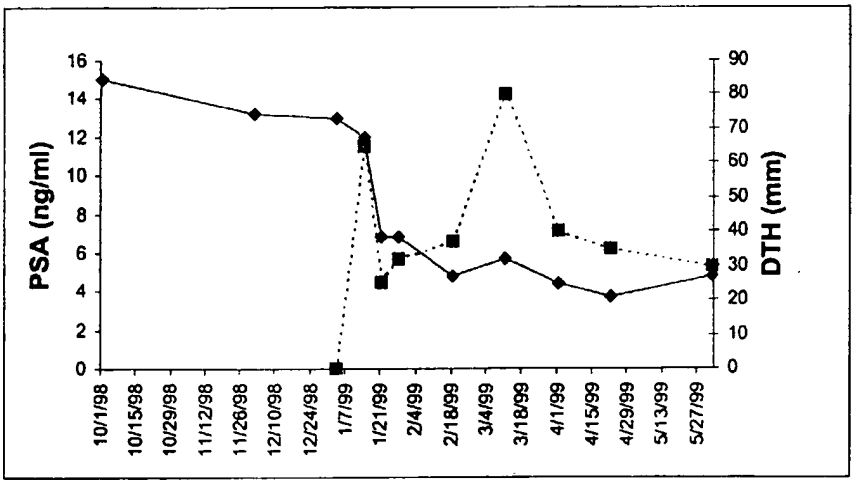

Fig. 6. PSA (◆) and DTH (■) values of patient 2.3 (age 64). Diagnosis in 11/98 with obstructive voiding symptoms, $T_{2b}N_0M_0$. Biopsy Gleason pattern 3–4. Immunotherapy was initiated in 1/1999. Currently, the patient has no obstructive voiding symptoms or any complaints. The gland has shrunk significantly. Only a small nodule in the left lobe is still detectable on DRE and on ultrasonography.

Patients on combined hormone and immune therapy: 4 patients were on combined hormone and immune therapy. One patient ($T_{4b}N_xM_{1b}$) did not respond to therapy. The other 3 had marked local improvement, decrease in bone pain (for those with bone metastases) and fall in PSA, although the concurrent hormone therapy renders these data difficult to interpret. However, the patient with lymph node involvement has been off hormone therapy for the last 7 months with no signs of disease recurrence (fig. 7).

Patients on immunotherapy only: 3 patients with bone metastases refused hormone therapy and were left on immunotherapy only. One of them is in stable condition, with PSA between 10 and 12, the bone pain has diminished, the local status has improved and he currently has no obstruc-

Table 1. Effect of combined hormone and immune therapy, or immune therapy only, on patients with prostate cancer

Patients with detectable lymph node or bone metastases				Patients with no detectable metastases			
combined hormone and immune therapy		immune therapy only		combined hormone and immune therapy		Immune therapy only	
responder	nonresponder	responder	nonresponder	responder	nonresponder	responder	nonresponder
2	5	1	2	7	0	5	4

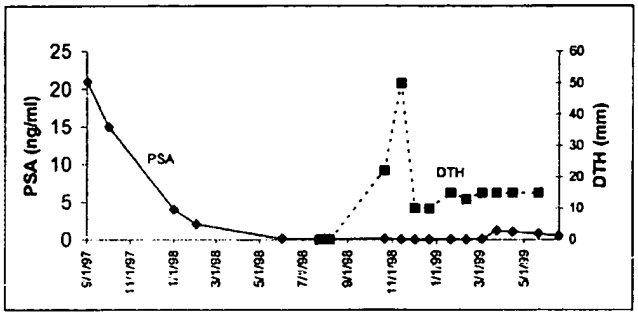

Fig. 7. PSA (◆) and DTH (■) values of patient 8 (age 69), diagnosed in 9/94. Transurethral resection of the prostate was performed in 9/94. Tumor recurrence in 97. Second transurethral resection of the prostate in 9/97. Gleason pattern 3–4. Distant lymph node involvement. Hormone therapy (Zoladex, Casodex) was started in 9/97, immunotherapy in 7/98. Hormone therapy was discontinued in 12/98. Currently no obstructive voiding symptoms, no prostate palpable on DRE and no lymph node involvement on CAT scan were noted.

tive voiding symptoms. The other 2 patients were not influenced by the therapy and are considered nonresponders.

In summary, 26 patients were included in the study, 10 with, 16 without detectable metastases (table 1). Fifteen patients improved following the therapy, 6 of them being solely on immunotherapy.

Discussion

Recently, interest in cancer immunotherapy has been reinvigorated with the appreciation that most human tumors encode tumor-associated antigens that can serve as potential targets for immune attack [8–19]. This factor, together with a recognition that the immunogenicity of a tumor may be enhanced by the addition of adjuvants, initially in the form of bacterial products [62], and now by recombinant factors [36], has opened a new chapter in a century-old saga [63] and has led to the development of new cellular or recombinant vaccines that await clinical testing.

One approach, the use of naked DNA (DNA that has been freed of all the proteins in the usual DNA-protein complexes) immunization, has recently received much attention [43–45]. DNA vaccines contain the gene or genes of an antigenic protein, such as a tumor-associated antigen. Host DC take up the foreign DNA and express the encoded protein inside the cell [64, 65]. An important advantage of this system is the fact that the expressed protein enters the MHC class I pathway of the cell [43–45]. The MHC class I molecules then carry peptide fragments of the encoded tumor-associated antigens to the cell surface where, by stimulating CD8+ cytotoxic T cells, they evoke cell-mediated immunity [65].

Recently, there have been reports of safe and successful plasmid DNA immunizations of humans against HIV [61] and malaria [46]. Alternatively, the DNA has been included into recombinant viral vectors such as vaccinia virus [22] or adenovirus [66]. Use of recombinant viral vectors ensures better penetration and intracellular expression of the gene of interest. Viral vectors also encode viral products, many of which can have an adjuvant effect [67].

Using PSMA as a target antigen, we have initiated a clinical trial for immunotherapy of prostate cancer. Unlike immunizations against foreign proteins, immunizations against self-antigens, including tumor-associated antigens, require the presence of self-reactive T lymphocytes and the breaking of self-tolerance [25]. The development of DTH following PSMA plasmid injection in all the patients included in our study shows that a robust cellular immune response against self-antigen in humans can be evoked.

PSMA is a hydrolase with the substrate and pharmacological characteristics of a neuropeptidase [68]. PSMA was originally considered to be restricted almost exclusively to prostate epithelial cells [69]. It is an integral, type II membrane protein and it is highly expressed in both normal and neoplastic prostate tissue and in prostate cancer metastases [69]. Recently, however, detectable PSMA levels have been found in tumor vascular endothelium, primary renal tumors, and, although at much lower density, in some normal tissues

such as duodenal mucosa and a subset of proximal renal tubules [70]. Using PSMA as a target for immunotherapy could be seriously offset by the development of serious toxicity such as autoimmunity. However, our trial, as well as other trials that target PSMA, has shown that the evoking of a T-cell immune response against PSMA does not lead to autoimmune reactions in other organs.

This is the third report on DNA vaccination in humans, but probably the first one that involves patients who were repeatedly immunized for more than 1 year [46, 61]. The primary objective of the study was to determine the safety of the PSMA vaccine after repeated intradermal injections. So far, almost 1.5 years since the study began, no patient has experienced any short- or long-term side effects, including anti-DNA antibody. The only exception is the development of fragile vasculature in the immediate vicinity of the prostate in 3 of the 4 patients who had immunotherapy prior to surgery. Additional studies are needed before the significance of this observation can be determined.

Recombinant human GM-CSF (rHuGM-CSF) has been found to be the principal mediator of proliferation, maturation, and migration of DC, important APC that play a major role in the induction of primary and secondary T-cell immune responses [36]. Similarly, expression of CD86 by DC is very important for the development of T cells, the likely effectors in antitumor immunity [31]. Local injection of rHuGM-CSF is expected to enhance vaccine immunogenicity and would likely be well tolerated based on clinical experience in other applications [36]. Our study demonstrates that repeated intradermal injection of rHuGM-CSF (Sargramostim) is a safe procedure and that in vivo transfection with CD86 is also well tolerated. On the other hand, soluble recombinant GM-CSF, as well as coexpression of CD86 with the target antigen, increases the likelihood for successful immunization. Use of a cocktail of a complex PSMA/CD86 plasmid and sGM-CSF leads to uniform immunizations in all patients.

Uniform and safe anti-PSMA immunization was also achieved with the use of the recombinant adenoviral vector. However, an important consideration is whether a preexisting adenovirus-specific immunity might compromise the ability of this vector to deliver antigen [66]. This concern, in view of the effectiveness of the complex plasmid plus sGM-CSF in immunizing 100% of the patients, has led us to the conclusion that the best priming for induction of anti-PSMA T cell immunity requires only the use of the PSMA/CD86 plasmid-sGM-CSF cocktail.

This was a phase I/II safety-dose escalation study. All patients became immunized against PSMA as demonstrated by the development of positive DTH reaction. The estimation of the biologic effectiveness of the treatment, however, was not straightforward. The patients were heterogeneous with regard to local advancement of disease, presence of distant metastases, of hormone treatment and refractoriness, which does not permit unequivocal interpretation of the results. Nevertheless, several responders to the immunotherapy could be identified.

The response rate was clearly dependent on the stage of the disease. Twelve of the 16 patients who had no evidence of distant metastases, including 5 on immunotherapy only, responded to the therapy with a drop in PSA levels and, whenever applicable, with improvement in local disease. In contrast, only 3 of the 10 patients with detectable distant metastases showed any improvement. It is possible that a large tumor load may have an anergizing effect on immune T cells [25, 32]. Progression of disease has also been associated with loss or decrease of class I expression by cancer cells. A complete loss of HLA class I has been reported to occur in 34% of primary prostate cancers and up to 80% in lymph node metastases [71]. When individual allelic expression was assessed, the minimum estimate of downregulation was up to 85% in the primary prostate cancers and almost 100% in the metastases [71]. Selective loss of some alleles of class I MHC may hamper T cell immunity, and at the same time the remaining HLA molecules may still bind to inhibitory receptors on natural killer cells thus blocking their cytotoxicity. An increasing tumor grade and dedifferentiation of tumor cells with loss of HLA molecules, PSA secretion and hormone dependence may be part of the natural course of the disease. Were this so, the adjuvant therapy in 3 of the 4 patients with advanced local disease, who received combined hormone and immune therapy prior to surgery, might have contributed to the selection of tumor cells that were both hormone refractory and MHC class I negative. Such 'natural' evolution of tumor during adjuvant treatment may explain the fast disease recurrence and the lack of response to immune therapy in these 3 patients. Downregulation or loss of HLA on tumor cells in metastases may be the reason that 7 of the 10 patients with metastatic disease in our study did not improve following treatment (table 1). Similar response rates, reported by others [41], may be the result of similar tumor evolution.

Downregulation or complete loss of HLA on tumor cells would require different approaches to immune therapy. Dedifferentiated tumor cells continue to express PSMA [69]. Use of the native molecule rather than peptides derived from it as a target, however, requires involvement of the humoral (antibody-mediated) arm of the immune system. In this respect, stimulating T cell immunity by naked DNA or viral immunization may be only the initial step. Ex-

perimental evidence from animals suggests that prior DNA immunization followed by boosts with soluble protein results in production of high titer, cytotoxic, antigen-specific antibodies [72].

In conclusion we have shown that: (1) repetitive DNA and recombinant adenoviral immunizations of humans are a safe procedure; (2) tolerance to self-antigens can be broken by immunizations with DNA that encodes the antigen, inserted in either a plasmid or a recombinant viral vector; addition of DNA encoding costimulatory molecules and soluble GM-CSF increases the immunization rate to 100%, and (3) the heterogeneity of the patients, especially in the presence of concomitant hormone therapy, does not permit unequivocal interpretation of the data with respect to the effectiveness of the therapy, but 6 of the 12 patients who were solely on immunotherapy could be identified as responders.

A phase II biological effectiveness clinical trial with a group of patients who have biochemical recurrence following radical prostatectomy is underway.

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